DOI: 10.22616/FoodBalt.2019.030

SHORT COMMUNICATION

THE EFFECT OF CONCENTRATED WHEY SOLIDS ON LACTOBIONIC ACID PRODUCTION BY *PSEUDOMONAS TAETROLENS*

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Abstract

Nowadays lactobionic acid production via microbial synthesis gain a high awareness. Lactobionic acid production by microbial pathway can be affected by various factors among them total solids in concentrated whey. The aim was to study the effect of acid whey permeate concentration on lactobionic acid production. The acid whey permeate was used as the study object. The total solids in acid whey was concentrated by the pilot scale FT22 Rising Film Evaporator (Armfield, UK). *Pseudomonas taetrolens* NCIB 9396 (NCTC, England) and *Pseudomonas taetrolens* DSM 21104 (DSMZ, Germany) were used for the study. The content of lactobionic acid (LBA) in the concentrated whey and control samples was determined using the high-performance liquid chromatography (Shimadzu LC 20 Prominence, Japan). The content of lactose in the acid whey and concentrated whey samples was determined using MilcoScan™ Mars (Foss, Denmark) and the high-performance liquid chromatography. The results showed that the highest yield of LBA was achieved at 20% of total solids content in the substrate. An increase of the total solids in the substrate more than 20% slows down the process due to the influence of minor whey compounds (as minerals and their salts) and higher lactose concentration reduces *Pseudomonas taetrolens* lactose dehydrogenase activity. The study results will help to improve an effective production of lactobionic acid by microbial pathway using acid whey.

Keywords: lactobionic acid, whey, *Pseudomonas taetrolens*, lactose oxidation

Introduction

Lactose (Lac) is known as milk sugar (4-O-b-D-galactopyranosyl-D-glucose) and is barely sweet (approximately 15% of sucrose), less soluble (solubility in water 195 g L^{-1}) than most of sugars (Seki, Saito, 2012; Gutiérrez et al., 2011; Schaafsma, 2008). These lactose properties deputize severe restrictions for its widespread use, so its separation or transformation into many products is preferable, like in value added products (Silva et al., 2015; Song et al., 2013; Seki, Saito, 2012). Lactobionic acid (LBA) is a compound produced via lactose oxidation and is known as aldonic acid (4-O-b-D-galactopyranosyl-D-gluconic acid), containing gluconic acid and galactose (Borodina, Mirgorod, 2014; Gutiérrez et al., 2012). The main characteristics of lactobionic acid include moisturising, antioxidant, acidifying and stabilising aptitude, and these properties have led to growing interest about the study of this polyhydroxy- acid inherent properties. It is wide used in cosmetic, food and medicine field due to lactobionic acid nontoxicity, amphiphilic, humectant, chelating, antioxidant, biocompatibility and biodegradability properties (Alonso et al., 2013a; Alonso et al., 2013b). *Pseudomonas taetrolens* shows high lactose bioconversion ability into lactobionic acid with no complicated nutrient requirements (Alonso et al., 2011).

During the last few years there have been several experiments to obtain lactobionic acid through biotechnological pathway with such a cheap feedstock as whey. Almost all available reports deal with the use of cheese whey as medium (Giorgi et al., 2018; Alonso et al., 2017). Acid whey (pH around 4.0 till 5.0) usage for the production of lactobionic acid has not yet been reported. Productivity of lactobionic acid by microbial synthesis can be affected by various factors and one of them is the total solids content in the substrate.

The aim was to study the effect of acid whey permeate concentration on lactobionic acid production.

These results will help to upgrade lactobionic acid production with *Pseudomonas taetrolens* DSM 21104 and NCIB 9396 using acid whey as a substrate.

Materials and Methods

Microorganisms and inoculum preparation

Freeze-dried *Pseudomonas taetrolens* NCIB 9396 culture was obtained from the England National Collection of Type Cultures (NCTC, England) and freeze-dried *Pseudomonas taetrolens* DSM 21104 culture from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) banks. *Pseudomonas taetrolens* strains were activated in nutrient broth agar (containing in g L^{-1}: 5 peptone, 1 meat extract, 2 yeast extract, 2.8 agar and 5 NaCl, respectively).

A 10 µL loopful of each strain was used to inoculate 100 mL of nutrient broth medium (containing in g L^{-1}: 5 NaCl, 5 peptone, 1 meat extract and 2 yeast extract). The precultured samples were incubated at 30 °C 16 h in an environmental shaker-incubator ES-20 at 220 rpm. Biomass of microorganisms was separated by 10 min centrifugation at 6000 rpm, using Hermle Labortechnik, Z 206 and further used as a bulk starter.

Acid whey

Acid whey permeate (producer SC "Tukuma piens") with following average composition: lactose

4.47±0.02%; proteins 0.38±0.01%, fats 0.01±0.01%, total solids 4.90±0.09% and pH 4.75±0.01 was used for the study.

The study design

The acid whey permeate was pasteurized 30 min at 95 °C temperature and further concentrated till solids content of 28.31±1.25% in the pilot scale FT22 Rising Film Evaporator (Armfield, UK).

Concentrated permeate was diluted with deionized water till 5%, 10% and 20% of total solids. Evaporator "Heidolph Laborota 4000 efficient" was used for permeate solids further concentration till 30% and 40%. The process was carried out at 60 °C, rotation speed of the flask 150 turns min^{-1}, pressure 40 to 85 mbar. The solids concentration was detected by a refractometer (Kruss, Germany). The strains were marked with letters N (sample contains *P. taetrolens* NCIB 9396) and D (sample contains *P. taetrolens* DSM 21104), but the samples with different solids concentration in such way –N5%, N10%, N20%, N30%, N40%, D5%, D10%, D20%, D30%, and D40%.

Each sample was inoculated with a 2% of *Pseudomonas taetrolens* bulk starter. The samples were cultivated on an environmental shaker-incubator ES-20 at 220 rpm and 30 °C for 48 h. Samples were withdrawn during cultivation for monitoring of substrate pH and colony forming units (CFU) of *Pseudomonas taetrolens* NCIB 9396 and DSM 21104. The amount of lactose was determined at the beginning and the end of the production process, but lactobionic acid at the end of the production process.

Analytical methods

Total plate count was determined using nutrient broth agar at 30 °C for 48 h and counted by the Acolyte colony counter (Model No:7510/SYN). Growth curves were depicted by plotting lg CFU mL^{-1} as a function of time. pH was measured using a pH electrode InLab® Expert Pro-ISM (METTLER TOLEDO, Switzerland). The content of lactose was determined in the acid whey and concentrated whey samples prior fermentation using MilcoScan™ Mars (Foss, Denmark). The concentration of lactobionic acid and residual lactose was analysed by high performance liquid chromatography HPLC (Prominance HPLC system, Shimadzu LC-20, Torrance, CA, USA). All samples were filtered before the analysis through a 2 µm filter paper and centrifuged to remove the cell debris and other water insoluble substances. Samples were centrifuged at 13,000 rpm 5 min. Detection of lactobionic acid was carried out in a refractive index detector RID-10A; YMC C18, 4.6 mm×250 mm, 5 µm column. Mobile phase isocratic elution on 2 L solution (14.36 g KH_2PO_4, 1.15 mL H_3PO_4, 20 mL acetonitrile and deionized water). Volume of injection sample 10 µL, flow rate 1 mL min^{-1}, temperature 40 °C. Detection of lactose was carried out in a detector DAD SPD-M20A; Alltech NH_2, 4.6 mm×250 mm, 5 µm column. Mobile phase isocratic elution (84% acetonitrile, 16% deionized water). Volume of injection sample 10 µL, flow rate 1 mL min^{-1}, temperature 35 °C. Samples were quantified according to HPLC-grade external analytical standards, lactobionic acid (Acrōs Organics), lactose (Sigma Aldrich).

Data analyses

Data acquisition and analysis were performed with Microsoft Excel 2010 programme. Statistical analyses were performed using Analysis of Variance (ANOVA) and t-test at significance level of $p<0.05$. All results are presented as the average of data from three independent experiments.

Results and Discussion

Substrates pH changes during lactose oxidation

During lactose oxidation process pH was analysed (Figure 1). Results showed that pH slowly increased during fermentation process. At the end of the process significant differences ($p<0.05$) were not established among samples N5% and D5%, N10% and D10%, N20% and D20%, N30% and D30%, N40% and D40%.

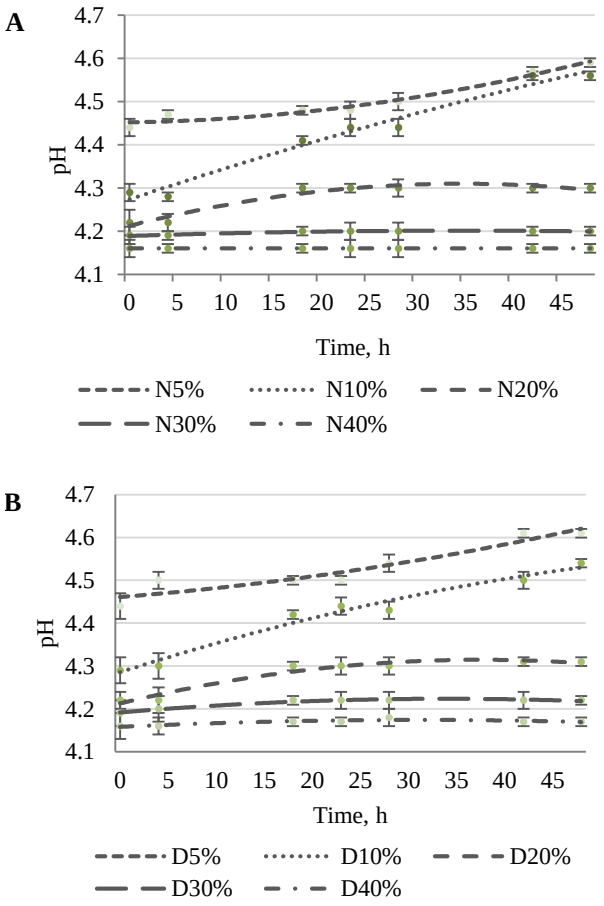

Figure 1. Time-course profile of pH changes in samples (A) containing *P. taetrolens* NCIB 9396 and (B) containing *P. taetrolens* DSM 21104 during cultivation at 30 °C for 48 h in an environmental shaker-incubator

It shows that both strains provide similar pH changes. At the beginning pH was different among samples, pH

increased with a decrease of the substrate solids concentration, respectively. We noticed that pH increased faster in samples with lower total solids concentration. pH stayed in the range from 4.16 till 4.61 in all samples. Giorgi et al. (2018) established that the lowest pH value of 4.2 was reached in lactose oxidation process with *Pseudomonas taetrolens* DSM 21104 using chemically pure lactose as a substrate. Alonso et al. (2017) observed the lowest pH value of 3.6 during shake-flask cultivation process of sweet whey permeate with *Pseudomonas taetrolens* LMG 2336 at 30 °C for 72 h. Usually pH decreases during lactose oxidation process in sweet whey with *Pseudomonas taetrolens*. The presence of nitrogen compounds in substrate promotes pH increasing in the range of 0.01 till 0.27. It could be explained with *Pseudomonas taetrolens* ability to produce proteins splitting enzymes (Alonso et al., 2011).

The study of Pseudomonas taetrolens microbial pathway

Pseudomonas taetrolens NCIB 9396 and DSM 21104 are grown in all samples during 48 h of incubation at 30 °C and reached around of 10^{10} CFU mL^{-1} (Figure 2) There were no significant differences among all samples at the end of cultivation process ($p<0.05$).

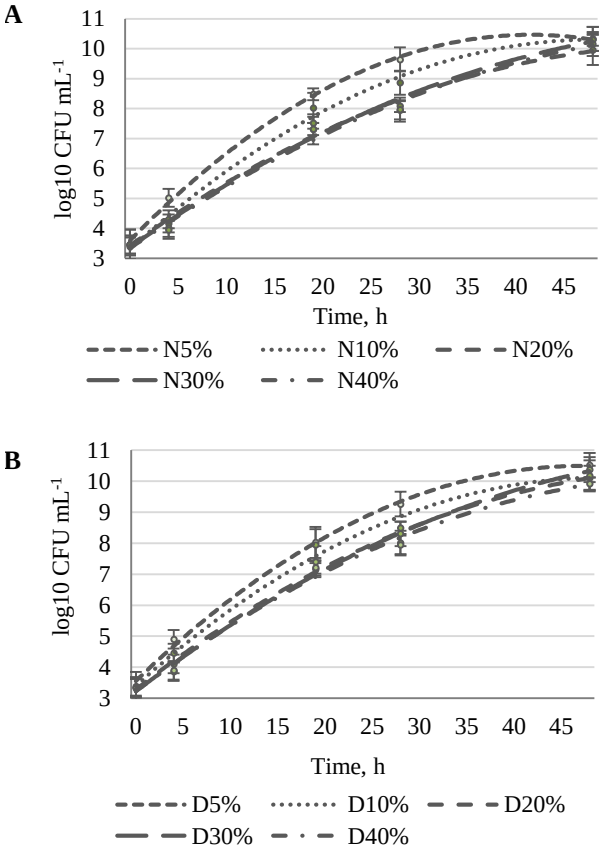

Figure 2. Time-course profile of CFU mL^{-1} changes in samples (A) containing *P. taetrolens* NCIB 9396 and (B) containing *P. taetrolens* DSM 21104 during cultivation in an environmental shaker-incubator

Pseudomonas taetrolens strains proliferate very similar in different acid whey permeate concentrations. Gorderska et al. (2014) determined that after 24 h of *Pseudomonas taetrolens* DSM 21104 cultivation in sweet whey (pH 6.5, amount of lactose 30 g L^{-1}) was reached around of 10^7 CFU mL^{-1} with 5% of inoculum in medium and 10^9 CFU mL^{-1} with 25% of inoculum in medium. Giorgi et al. (2018) established that maximum cell density of up to 10^9 CFU mL^{-1} was achieved after 48 h of incubation with *Pseudomonas taetrolens* DSM 21104 in substrate that contains 10 g L^{-1} lactose.
In Figure 2 we can observe that *Pseudomonas taetrolens* had grown faster in the substrates with 5% and 10% of total solids, but amount of CFU mL^{-1} were the same in all samples at the end of fermentation. It shows that *Pseudomonas teatrolens* needs more time to adopt in samples with higher solids concentration.

Lactobionic acid yield

The higher lactobionic acid conversion yield was reached in samples N5%, D5%. N10%, D10%, N20% and D20% (see Table 1). The significant differences were not established among these samples ($p<0.05$).

Table 1

Lactose (Lac) and Lactobionic acid (LAB) yield in end of 48 h production process

Sample	Lac, g L^{-1}	LBA, g L^{-1}	Conversion yield, %*
N5%	37.4±2.1	9.1±1.2	20.0±1.3[a]
D5%	38.0±1.8	8.5±1.2	18.8±1.9[a]
N10%	65.0±2.6	17.2±0.8	20.4±1.4[a]
D10%	65.8±1.2	16.9±1.4	20.0±1.2[a]
N20%	148.1±3.2	39.8±0.9	21.8±1.7[a]
D20%	152.2±1.9	41.3±1.8	22.6±1.9[a]
N30%	229.2±2.4	46.1±0.6	17.2±1.1[b]
D30%	231.0±2.1	43.9±1.3	16.4±1.3[b]
N40%	315.5±2.1	51.8±1.2	13.9±1.1[c]
D40%	302.0±2.3	58.6±1.3	15.8±1.1[bc]

*Yield was shown as the % of lactose converted into lactobionic acid after 48 h.
Results indicated with the same letter do not differ significantly ($p<0.05$).

Results showed that lactobionic acid conversion yield is lower in acid whey permeate samples with total solids higher than 20%.
Pleissner et al. (2017) has mentioned that the production of lactobionic acid increased with the increase of whey concentration. In an experiment with sample containing 200 g L^{-1} lactose (pH above 5.0 during fermentation) was produced 197 g L^{-1} of lactobionic acid after 180 h of cultivation with *Pseudomonas taetrolens*. Miyamoto et al. (2000) has established that the production rate of LAB with *Pseudomanas taetrolens* was higher in samples with 150 g L^{-1} than 200 g L^{-1} lactose. Murakami et al. (2003) has concluded that high solids concentration in whey may decrease the penetration rate of lactose through microorganism cell membrane or high amount of total solids in substrate can cause inhibition of the microorganism produced enzymes.
Goderska et al. (2014) gained 15.79 g L^{-1} lactobionic acid from 30.29 g L^{-1} lactose after 50 h of cultivation

process with *Pseudomonas taetrolens* at 30 °C. Mayamoto et al. (2000) reached 90% of lactobionic acid from lactose with *Pseudomonas taetrolens*. Alonso et al. (2012) reached 100% of lactobionic acid yield from sweet whey after 60 h of cultivation with *Pseudomonas taetrolens*. Seems like acid medium hinders *Pseudomonas taetrolens* ability to convert lactose to lactobionic acid. It could be explained with *Pseudomonas taetrolens* lactose dehydrogenase composition, containing flavin adenine dinucleotide as a prosthetic group. This flavoprotein does not use oxygen as direct electron acceptor and presents an optimum pH at 5.6. Lactose is converted by lactose oxidase to lactobiono-δ-lactone and then by lactonase in lactobionic acid. Lactonase presents an optimum at pH 6.5–6.7 (Alonso et al., 2013b). Low acid whey pH is the reason why the conversion yield is not reached as high as it is in other researches, where sweet whey was used as a substrate.

Conclusions

The most suitable acid whey permeate concentration is up to 20% for lactose oxidation with *Pseudomonas taetrolens* NCIB 9396 and DSM 21104. Low acid whey pH is the reason why the conversion yield is not reached as high as it is in other researches. The study suggests to adjust the acid whey pH prior lactose oxidation with *Pseudomonas taetrolens* and to prolong cultivation time.

Acknowledgment

Research was funded by the grant „Strengthening Research Capacity in the Latvia University of Life Sciences and Technologies" project Nº Z23.
We acknowledge Dr.sc.ing. Ruta Galoburda and Dr.sc.ing. Liga Skudra for valuable consultation.

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